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Complexing of heparin with phosphatidylcholine

A possible supramolecular assembly of plasma heparin

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In a series of attempts to reveal plasma heparin, we found that high ionic strength and modification of protein amino groups were not effective in extracting endogenous heparin (or, indeed, a large percentage of exogenous labelled heparin), whereas delipidation in the presence of 4M-guanidinium chloride gave high yields, indicating that plasma heparin may be assembled with compounds other than proteins, in a form making it inaccessible to water and ions. During the extraction of lipids, a paradoxical entry of heparin into the organic phase was observed. Detergents, including sodium dodecyl sulphate, did not shift heparin into the aqueous phase, whereas repeated chloroform/methanol extraction did so. Using purified compounds we were able to reproduce *in vitro* both the scavenging of heparin from water as well as the formation of heparin–phosphatidylcholine complexes soluble in organic solvents. Evidence for complexing of heparin with phosphatidylcholine was also obtained by electrophoretic and ultracentrifugation assays. The quaternary-ammonium-containing phosphatidylcholine was the more effective phospholipid in binding heparin; anionic phospholipids did not bind. Only heparin-like glycosaminoglycans bound phosphatidylcholine, but less-sulphated compounds (heparan sulphate and dermatan sulphate) were weaker ligands. Gel-filtration experiments showed that heparin was not bound to liposome vesicles, but that a measurable percentage of the phospholipids was stripped off from vesicles and was found in the form of a complex separable from liposomes by gel filtration. The molecular basis as well as the biological role of the interaction of heparin with major membrane phospholipids are discussed.

Heparin is a powerful effector in the vascular system that is produced by mast cells, perivascular elements usually found close to venous capillaries. However, it is not known whether and in what molecular form heparin enters the bloodstream.

Endogenous plasma heparin has hardly been investigated, and only indirect evidence for its presence has been obtained so far: thus Horner (1975) demonstrated a heparin-like material labelled with radioactive sulphate associated with circulating cells, including platelets, and human platelets have been shown to be able to release heparin *in vitro* (Vannucchi *et al.*, 1982). In addition, a demonstration of the existence of endogenous heparin in human plasma on the basis of isotope-dilution assays has been claimed (Dawes & Pepper, 1982).

Heparin is a well-known protein ligand (Lindahl & Höök, 1978), and the difficulties as well as the discrepancies in revealing endogenous heparin were generally ascribed to this kind of interaction. In the present paper we provide data indicating that compounds other than proteins must be considered: a complex of heparin with phosphatidylcholine was identified after incubation of heparin with human plasma; furthermore, the ability of phosphatidylcholine to abstract heparin from aqueous solutions was demonstrated.

Materials and methods

Materials

Radioactive phosphatidylcholine (1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine; sp. radioactivity 57mCi/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Radioactive

heparin ([^{3}H]acetyl-heparin; sp. radioactivity 35×10^4 d.p.m./μg) was generously given by Professor Ulf Lindahl (Uppsala, Sweden). Chromogenic substrates (2238 and 2222) were from Kabi (Stockholm, Sweden).

Special reagents, including glycosaminoglycans and various phospholipids, were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) Standard, unlabelled, heparin was a gift from Dr. Pietro Bianchini (Opocrin, Modena, Italy). Sepharose CL-6B was from Pharmacia (Uppsala, Sweden). All the other reagents were from Carlo Erba (Milano, Italy).

Plasma sampling

Blood samples were collected from human healthy volunteers, with 0.38% trisodium citrate as anticoagulant. Plasma was obtained by centrifuging the blood at 860*g* for 15min, and the supernatant was further centrifuged at 3000*g* for 10min to remove platelets. Samples were stored at −20°C until used and stocked in 1ml portions.

Extraction of endogenous and exogenous heparin from plasma samples

(*a*) *Treatment with trichloroacetic acid and ethanol.* An amount of radioactive heparin corresponding to 1×10^5 d.p.m. was added to 10ml of plasma. Trichloroacetic acid was added to reach a final concentration of 10% (w/v) and the sample was centrifuged at 10000*g* for 10min. To the supernatant was added 2.5vol. of cold ethanol and the mixture was stored at 4°C overnight. After centrifugation the sediment was counted for radioactivity in a Packard Isocap liquid-scintillation spectrometer. Parallel samples without exogenous heparin were checked for the presence of endogenous heparin by electrophoresis as indicated below.

(*b*) *DEAE-Sephacel chromatography.* A 10ml portion of human plasma (with and without exogenous labelled heparin) was passed through a column (1cm × 3cm) of DEAE-Sephacel. The column was washed with 0.6M-NaCl in 0.05M-sodium acetate buffer, pH4.0, and then eluted with the same buffer containing 2.0M-NaCl. After dialysis and freeze-drying, samples were either counted for radioactivity in a liquid-scintillation spectrometer or checked for the presence of endogenous heparin as indicated below.

(*c*) *Modification of amino groups of proteins with paraformaldehyde/cyclohexane-1,2-dione.* To a 10ml portion of human plasma was added 1×10^5 d.p.m. of labelled heparin. The plasma then was diluted with an equal volume of 4% (w/v) paraformaldehyde in phosphate-buffered saline (0.15M-NaCl/0.01M-sodium phosphate buffer, pH7.4) and stored for 12h at 4°C. The samples were then dialysed against cyclohexane-1,2-dione as described by Rosenberg & Damus (1973). The dialysed samples were submitted to DEAE-Sephacel chromatography as described above. Parallel samples (without radioactive heparin) were subjected to an identical procedure, and the presence of endogenous heparin was checked by electrophoresis.

(*d*) *Density-gradient centrifugation.* To a 5ml portion of plasma (with or without labelled heparin) was added 8M-guanidinium chloride/0.2M-Tris/HCl buffer, pH8, and solid CsCl to reach a final concentration of 4M-guanidinium chloride and a final density of 1.5g/ml. The solution was then centrifuged at 100000*g* for 48h at room temperature. The bottom of the tube was pierced and 1ml fractions were collected. Fractions containing labelled heparin were counted for radioactivity, and unlabelled samples were dialysed, dried and checked for the presence of heparin by electrophoresis as well as by anticoagulant assays.

(*e*) *Extraction procedure of plasma for the unmasking of endogenous heparin: Folch extraction and treatment with guanidinium chloride and* $(NH_4)_2SO_4$. A 1ml portion of human plasma was diluted 1:1 (v/v) with 8M-guanidinium chloride. Then 19vol. of chloroform/methanol (2:1, v/v) was added to each sample and the mixture was shaken at room temperature for 1h. Formation of separate phases was induced by adding 10ml of 4M-guanidinium chloride. The upper phase was collected after centrifugation, and the lower phase was re-extracted with 4M-guanidinium chloride/methanol (2:1, v/v). The two aqueous phases were mixed together and evaporated to dryness *in vacuo*, and then dissolved in 5ml of distilled water. Next 4vol. of saturated $(NH_4)_2SO_4$ adjusted to pH10.0 with conc. NH_3 was added, and the residual proteins were coagulated overnight at 70°C. The precipitate was removed by filtration on glass-fibre filters (Whatman GF/G), and the filtrate was dialysed and freeze-dried. Radioactive heparin was used as an internal standard to check the extraction. Also, to another 1ml portion of plasma was added radioactive heparin (1×10^5 c.p.m.) and the mixture was subjected to the procedure described above as well as to the unmodified Folch extraction (i.e. without addition of guanidinium chloride). Samples of the organic, the aqueous and the interfacial phases were taken and counted for radioactivity in a liquid-scintillation spectrometer.

Identification and characterization of endogenous heparin

Aqueous phases obtained by the extraction procedure described above were evaporated to dryness *in vacuo* and dissolved in 50μl of distilled

water, and 2μl portions were analysed by electrophoresis by the procedure of Cappelletti *et al.* (1980). The characterization of heparin was based on the following criteria: (*a*) co-migration with authentic standards of heparin at pH 5.0 in ethanol-containing buffers in the presence of Ba^{2+} ions as well as co-migration with standard heparin at pH 1.0 in 0.1M-HCl; (*b*) sensitivity to degradation with nitrous acid; (*c*) anticoagulant activity as revealed by the Activated Partial Thromboplastin Time (Basu *et al.*, 1972) and with the chromogenic substrates 2238 and 2222 (from Kabi) to measure the antithrombin III-mediated inhibition of thrombin and of activated Factor X, measured in accordance with the instructions of the manufacturer.

Purification of endogenous heparin by affinity chromatography on antithrombin III–agarose

Glycosaminoglycans were loaded on to a gel of antithrombin III–Sepharose 4B (Pharmacia) prepared in accordance with the instructions of the manufacturer: 10mg of antithrombin III (a gift from Professor B. Casu) was coupled to 10ml of agarose. The column was washed with 50ml (7 column volumes) of 1M-Tris/HCl buffer, pH 7.4; retained glycosaminoglycans were eluted with 30ml of 1.5M-NaCl in 0.1M-Tris/HCl buffer, pH 7.4. Both elution volumes were dialysed against distilled water and freeze-dried for the analysis of glycosaminoglycans.

Preparation of phosphatidylcholine liposomes

Liposomes were prepared by sonication of a suspension of pure phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; Sigma Chemical Co.) at a concentration of 5mg/ml in Tris/HCl buffer, pH 7.2, in accordance with Johnson & Bangham (1969). The homogeneity of the preparation was assayed by electron microscopy: the average diameter of the vesicles was about 25–30nm. The suspension was stable at 0°C for at least 48h.

Sepharose CL-6B chromatography of liposomes and heparin

Of various molecular sieves tested, Sepharose CL-6B was the most efficient for the separation of phosphatidylcholine liposomes of an average diameter of 25nm from 10000–13000-M_r heparin. A 1cm × 100cm column of Sepharose CL-6B was loaded and equilibrated with 0.005M-Tris/HCl buffer, pH 7.4. To liposome suspensions corresponding to 25mg of phosphatidylcholine were added 2×10^5 d.p.m. of radioactive phosphatidylcholine and 1×10^5 d.p.m. of radioactive heparin as described in the legends to the Figures. Elution was carried out at a flow rate of 4ml/h with an LKB peristaltic pump. Fractions (2ml) were collected with a Isco–Foxy fraction collector.

Results

The precipitation of protein by trichloroacetic acid and in turn of trichloroacetic acid-soluble compounds with ethanol is a widely used procedure to extract glycosaminoglycans from biological materials. This method was ineffective for the isolation of endogenous or exogenous heparin from human plasma. A check with radioactive heparin showed that the latter was co-precipitated with proteins and was almost completely lost. Density-gradient centrifugation in saturated CsCl in the presence of guanidinium chloride is an effective strategy for the isolation of exogenous heparin, but it was ineffective for the endogenous counterpart. A similar result was obtained when the amino groups of proteins were modified and the material was chromatographed on DEAE-cellulose (results not shown).

The effectiveness of modification of protein for the extraction of exogenous heparin and the failure of the same procedure to reveal any endogenous heparin led us to conclude that endogenous heparin, when present, must be assembled in a different molecular form, compared with the exogenous counterpart, and that compounds other than proteins must be involved in this complexing.

Plasma was thus approached in terms of lipid chemistry, with a look at the distribution of radioactive exogenous heparin. Table 1 shows that heparin was distributed between the aqueous and the organic phases of the Folch extraction (Folch *et al.*, 1957), after incubation with plasma.

The percentage distribution in the aqueous phase was strongly influenced by the presence of guanidinium chloride, which prevents the complexing of heparin with the interfacial material.

The extraction of plasma with 19vol. of chloroform/methanol (2:1, v/v) by the Folch *et al.* (1957) procedure in the presence of 4M-guanidinium chloride was also effective in revealing endogenous heparin. In 20 extraction experiments (carried out with samples from healthy donors never treated with heparin) we were able to obtain a high percentage of positive results (75%). The failure to obtain a 100% reproducibility could be due to the different concentrations of heparin in the blood of different donors. Indeed, the amounts of heparin within the group of positive samples (assayed with the chromogenic substrate of thrombin, 2238) varied widely (from 4 to 30i.u./ml of plasma).

However, even different extraction experiments with ten samples from the same donor gave only 80% positive results with regard to the presence of heparin in the aqueous extract. Some samples,

Table 1. *Distribution of ratioactive heparin between the aqueous and the organic phase after chloroform/methanol extraction*
To human plasma was added radioactive heparin (1×10^5 c.p.m. per 1 ml of plasma) and the mixture was incubated overnight. The samples were extracted with 19 vol. of chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957). The same amounts of radioactive heparin, dissolved in 1 ml of water or in 1 ml of plasma to which guanidinium chloride had been added give a final concentration of 4M, were also subjected to the same extraction procedure. Radioactivity of the three phases was counted by liquid scintillation. The values represent ranges of results from ten experiments.

Experimental conditions	Radioactivity (% of input)		
	Aqueous phase	Interface	Organic phase
Heparin	100	—	—
Heparin/plasma	15–20	60–80	10–20
Heparin/plasma/4M-guanidinium chloride	60–80	15–30	0–10

Table 2. *Concentrations of plasma heparin as measured with different coagulometric assays*
The Table gives the calculated amounts of endogenous heparin extracted from three different samples of human plasma with the Folch/guanidine extraction strategy as measured with different assays. Assays with chromogenic substrates 2238 and 2222 were carried out as described by Teien *et al.* (1976), assays with Factor X-deficient plasma as described by Yin *et al.* (1973), and determinations of the Activated Partial Thromboplastin Time as described by Basu *et al.* (1972).

Type of assay	Calculated amount of heparin (μg/ml of plasma)		
	Sample A	Sample B	Sample C
2238, chromogenic (thrombin)	5.3	1.5	7.4
2222, chromogenic (activated Factor X)	10.8	19.5	23.8
Yin–Wessler (activated Factor X, coagulometric)	17.0	46.7	9.5
Activated Partial Thromboplastin Time	2.5	1.75	9.17

which were negative with respect to the aqueous phase, were positive in the organic extract. Repeated extraction of the dried material recovered from the organic phase with the same procedure progressively cleaned heparin from unknown ligands (presumably lipids), shifting the former into the aqueous phase. When plasma proteins were precipitated with 10 vol. of ethanol/diethyl ether (2:1, v/v) and the supernatant was dried and analysed on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, not a single band of compounds positive for protein staining was revealed, whereas high-M_r aggregates were revealed by Toluidine Blue staining (Fig. 1). The aggregates were radioactive when [^{14}C]phosphatidylcholine (or [^{3}H]heparin) had been incubated with the plasma samples overnight beforehand: this result indicates a complexing of heparin with lipids, and the complex formed seems to be resistant to sodium dodecyl sulphate.

The water-soluble endogenous heparin was characterized by electrophoresis in terms of co-migration with authentic standards at different pH values (pH 1.0 and pH 5.0) and by sensitivity to nitrous acid degradation (Cappelletti *et al.*, 1980). The results of the electrophoretic analysis are shown in Fig. 2: authentic heparin is present together with other glycosaminoglycans. Endogenous heparin was also characterized in terms of anticoagulant activity with various assays (see Table 2): the ratio between antithrombin and anti-Factor Xa activities indicates a relatively low M_r value (Lindahl & Höök, 1978).

The results reported above, which suggest but do not prove interaction of plasma heparin with lipids, led us to plan experiments with purified reagents to see whether a clear-cut interaction between heparin and lipids was actually occurring. Phospholipids, especially those containing cationic groups, were considered the best candidates as ligands. Heparin was incubated *in vitro* with liposome vesicles obtained by sonication of different phospholipids in aqueous buffers.

The metachromasia of the suspension was checked at various time intervals, and a time-dependent disappearance of the Toluidine Blue metachromasia was observed. The time course was slow, and phosphatidylcholine turned out to be the most effective 'metachromasia scavenger', compared with other phospholipids (Fig. 3). The interaction of heparin with phospholipids in water was also studied by electrophoretic assays. Heparin was incubated with suspensions of liposomes prepared from various phospholipids, the mixtures

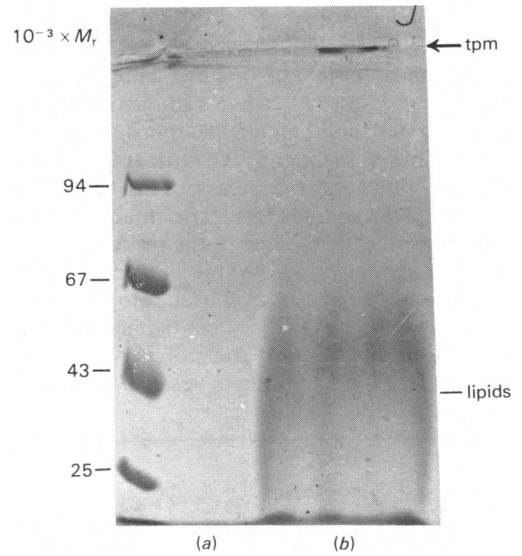

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of an ethanol/ether extract of human plasma*

Total lipids were extracted from 5ml of human plasma with 5vol. of ethanol/diethyl ether (2:1, v/v); the proteins were precipitated by centrifugation at 500*g* for 30min, and the supernatant was evaporated to dryness *in vacuo*. The dried material was solubilized in sample buffer containing sodium dodecyl sulphate and subjected to polyacrylamide-gel electrophoresis in accordance with the procedure of Laemmli (1970). The gels were stained with 1% Toluidine Blue after washing of the sodium dodecyl sulphate from the gel. After identification of the Toluidine Blue-positive bands, the gel was stained with 1% Coomassie Brilliant Blue to reveal standard proteins of known M_r as well as other proteinaceous material present in the ethanol/ether extract. (*a*) Standard proteins: phosphorylase *a* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000) and chymotrypsinogen A (M_r 25000). (*b*) Ethanol/ether extract; tpm indicates Toluidine Blue-3 positive material.

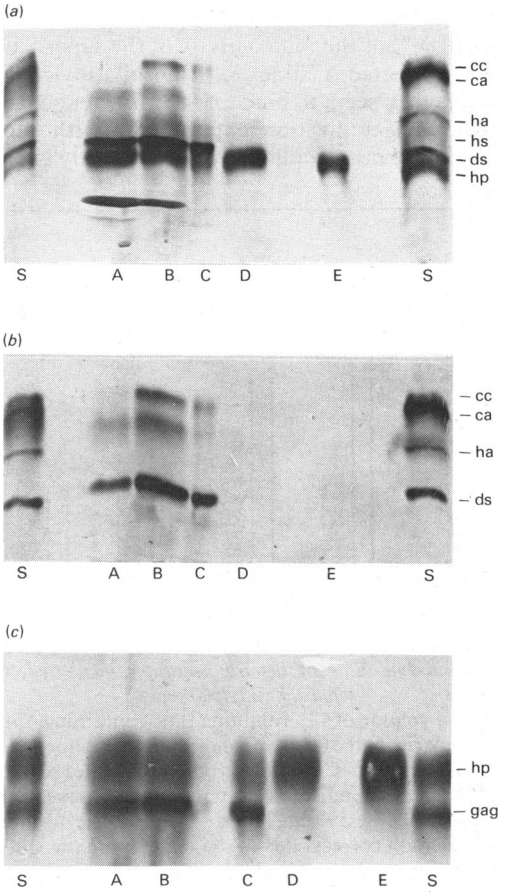

Fig. 2. *Characterization of endogenous heparin from human plasma*

Details of the extraction procedures and cellulose acetate electrophoresis are given in the Materials and methods section. (*a*) Electrophoresis at pH5.0. (*b*) Electrophoresis at pH5.0 after nitrous acid treatment. (*c*) Electrophoresis at pH1.0. Key: gag, glycosaminoglycans; S, standard glycosaminoglycan mixture including chondroitin sulphate C (cc), chondroitin sulphate A (ca), hyaluronic acid (ha), heparan sulphate (hs), dermatan sulphate (ds) and heparin (hp); A and B, glycosaminogycans extracted from the aqueous phases of the Folch/guanidinium extract of plasma samples from different donors; C, glycosaminoglycans extracted from the choloroform phase of sample A; D, sample A after purification of heparin by affinity chromatography on antithrombin III–Sepharose; E, standard heparin.

were centrifuged and the supernatants were analysed by electrophoresis. Phosphatidylcholine was able to abstract heparin from water, but anionic phospholipids were not (results not shown). These results led us to continue our studies by focusing only on phosphatidylcholine, which is the major phospholipid of the cell membrane and, in our hands, the most efficient ligand of heparin.

Phosphatidylcholine was incubated with various glycosaminoglycans, and heparin was completely abstracted at a lipid/glycosaminoglycan ratio of 50:1 (w/w), whereas only 15% of the co-polymeric glycosaminoglycans heparan sulphate and dermatan sulphate were abstracted when incubated with lipids at the same molecular ratio (Fig. 4). On incubation of heparin with a lower ratio of phosphatidylcholine (20:1), a progressive lowering of the electrophoretic mobility of heparin was observed (Fig. 5). When radioactive heparin was incubated with phosphatidylcholine liposomes and

the latter were sedimented by prolonged centrifugation, 80% of the radioactivity of the supernatant was abstracted (Table 3). Phosphatidylcholine vesicles thus seem to bind and to abstract heparin. However, gel-filtration experiments with radioactive heparin and radioactive phosphatidylcholine showed that a more complex interaction occurs (Fig. 6). We selected a molecular sieve able to separate phosphatidylcholine liposomes from heparin (Figs. 6*a* and 6*b*); when radioactive heparin was incubated with unlabelled phosphatidylcholine we found a shoulder of the radioactive heparin peak that indicates an increase in molecular mass (Fig. 6*c*). The counterpart experiment of radioactive phosphatidylcholine in the presence of heparin showed that a measurable percentage of the liposome radioactivity was shifted into the heparin peak (Fig. 6*d*). The results of centrifugation and of gel-filtration experiments are not in strict agreement: rather than a binding to vesicles (as reported

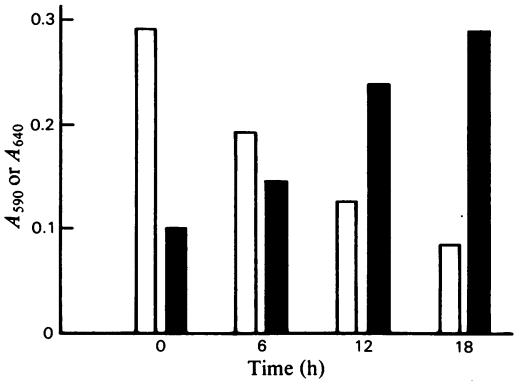

Fig. 3. *Time course of the disappearance of metachromasia with Toluidine Blue of heparin solutions incubated with phosphatidylcholine*

A 1 ml volume of 1% Toluidine Blue containing 4 μg of heparin and 100 μg of phosphatidylcholine was incubated at 37°C in a spectrometer cuvette. At the time intervals indicated the cuvette was assayed for absorbance at 590nm and at 640nm. The former wavelength corresponds to the metachromatic shift to the red of the dye in the presence of heparin. □, A_{590}; ■, A_{640}.

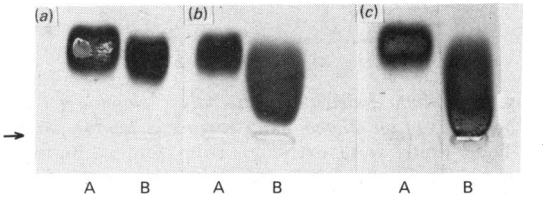

Fig. 5. *Time course of the lowering of the electrophoretic mobility of heparin after incubation with phosphatidylcholine*

(*a*) Immediately after the addition of phosphatidylcholine; (*b*) after 12h incubation at 37°C; (*c*) after 48h incubation at 37°C. Key: A, standard heparin without phosphatidylcholine; B, standard heparin incubated with phosphatidylcholine. Electrophoretic conditions were as indicated in the text.

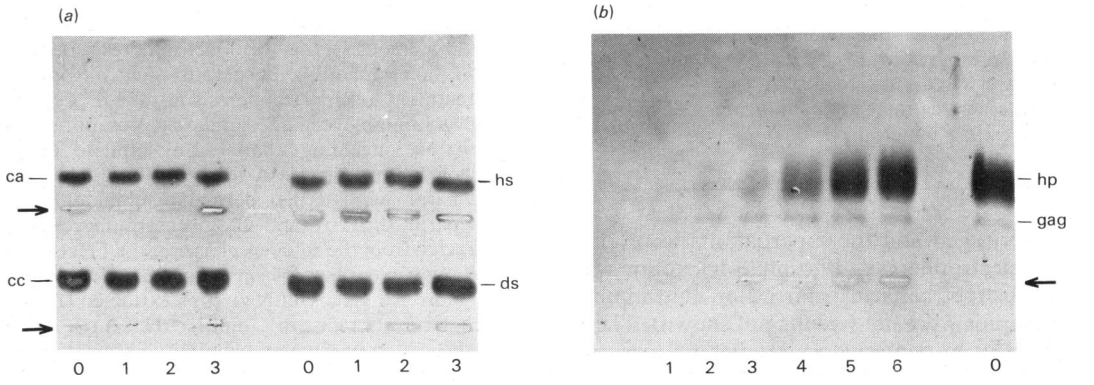

Fig. 4. *Specific abstraction of heparin from an aqueous solution by phosphatidylcholine as revealed by cellulose acetate electrophoresis*

The indicated glycosaminoglycans (for abbreviations see the legend to Fig. 2) were incubated overnight with phosphatidylcholine liposomes prepared as indicated in the Materials and methods section. The numbers under electrophoresis lanes indicate phosphatidylcholine/glycosaminoglycan concentration ratios: 0, no phosphatidylcholine; 1, 50:1; 2, 25:1; 3, 12:1; 4, 6:1; 5, 3:1; 6, 1:1. The samples were centrifuged at 30000*g* for 1 h, and portions of the supernatants were applied on cellulose acetate sheets and run at pH 1.0 as described in the Materials and methods section. (*a*) shows that chondroitin sulphates were not scavenged as well as heparan sulphate and dermatan sulphate. (*b*) shows a nearly complete abstraction of heparin starting from the ratio 25:1; while the heparin band disappears, the band of the other contaminating glycosaminoglycans is still evident.

Table 3. (*a*) *Complexing of heparin with phosphatidylcholine as revealed by ultracentrifugation and* (*b*) *solubility of radioactive heparin in organic solvents after incubation with phosphatidylcholine*

For the experiments reported in (*a*) radioactive heparin (1×10^5 d.p.m.) was solubilized in 50mM-Tris/HCl buffer, pH 7.2, and incubated with and without liposome suspension (final concn. 0.5mg of phosphatidylcholine/ml) for 24h at 37°C. The incubation mixtures were centrifuged at 40000*g* for 2h. The sediment and the supernatant were solubilized in a scintillation cocktail and their radioactivities counted by liquid scintillation. For the experiments reported in (*b*) the same amount of radioactive heparin was incubated in distilled water overnight in the absence and in the presence of phosphatidylcholine (400μg/ml). Samples (1ml) were dried and induced to solubilization in the same volume of pure chloroform by vigorous shaking with a vortex. The samples were centrifuged at 10000*g* for 15min, and the radioactivities of the supernatant as well as the sediment were separately counted by liquid scintillation. The values are the means of two experiments.

(*a*) Complexing of heparin with phosphatidylcholine

	Radioactivity (c.p.m.)	
	Sediment	Supernatant
Heparin	340±60	27890±3567
Heparin + phosphatidylcholine	28870±2546	3890±430

(*b*) Solubility of heparin in organic solvents

	Radioactivity (c.p.m.)	
	Soluble	Insoluble
Heparin	1200±51	80560±890
Heparin + phosphatidylcholine	61550±580	21678±1589

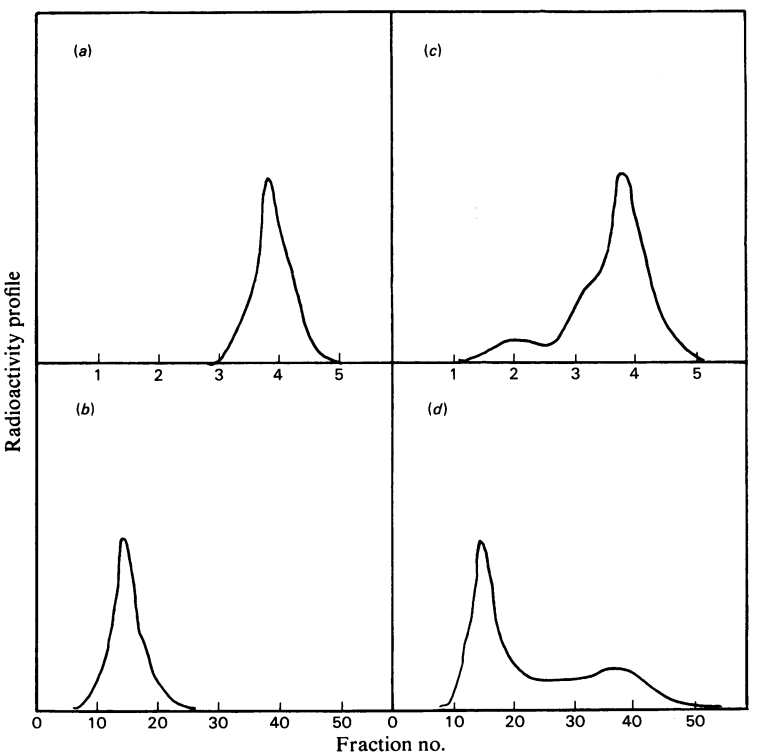

Fig. 6. *Complexing of heparin with phosphatidylcholine as revealed by gel filtration*

Sepharose CL-6B chromatography was carried out as indicated in the Materials and methods section. Elution profiles: (*a*) radioactive heparin; (*b*) radioactive phosphatidylcholine: (*c*) radioactive heparin incubated with unlabelled phosphatidylcholine; (*d*) radioactive phosphatidylcholine incubated with unlabelled heparin. The phosphatidylcholine/heparin ratio was 50:1 in both (*c*) and (*d*) experiments.

by other authors: see the Discussion section), it seems that heparin abstracts phosphatidylcholine, presumably by stripping off the outer phospholipid layer of the liposomes.

This particular supramolecular assembly of heparin with phosphatidylcholine seems also to be resistant to organic solvents, as shown in Table 3, where the transfer of heparin (radioactive) into the chloroform phase after incubation with liposomes is shown. A similar result was obtained when the entry of heparin into the chloroform phase was checked by electrophoresis (not shown).

Discussion

The data obtained in this study confirm the presence of heparin in human plasma as previously reported (Horner, 1975; Dawes & Pepper, 1982). They also indicate that plasma heparin is not the free compound, but that it interacts with other components of plasma, hampering its extraction by conventional glycosaminoglycan biochemistry. Heparin is well known to be a very avid ligand of a number of plasma proteins (see Lindahl & Höök, 1978, for review); however, the failure to obtain endogenous heparin with high concentrations of guanidinium chloride, or after modification of the amino groups of proteins, indicates that interaction with proteins is not mainly responsible for the failure to extract endogenous heparin. Rather, our results demonstrate that delipidation is a critical step required to reveal plasma heparin, suggesting that the latter is transported in plasma as a lipopolysaccharide complex at least partially excluded from the water domain. Proteins could be also involved in the formation of the complex. The variable but relatively large amount of heparin extractable from human plasma is an indirect proof that endogenous heparin is a masked compound, inactive as anticoagulant. As a matter of fact, exogenous heparin renders plasma uncoagulable at nanomolar concentrations, but we extracted micromolar concentrations of endogenous heparin from the blood of healthy donors with no defect of coagulation. It is not known how and whether endogenous heparin is recoverable from this complex to restore its anticoagulant activity in the bloodstream.

The experiments with purified compounds showed that heparin does indeed bind phosphatidylcholine. However, the nature of the interaction is not yet clear. Patel *et al.* (1983) showed a lytic effect of concentrated heparin on phosphatidylcholine-containing liposomes, and they also claim a binding to vesicles on the basis of centrifugation experiments. We also obtained positive results of binding to vesicles by centrifugation (see Table 3), but we did not do so with the experiments of gel filtration (see Fig. 6), i.e. we were unable to demonstrate a co-elution of heparin with the large liposome peak. It seems more likely that heparin strips off phosphatidylcholine from vesicles, in accord with the lytic effect reported by Patel *et al.* (1983), operating as the nucleus of assembly of smaller-size complexes. These complexes are co-sedimented with liposomes by ultracentrifugation, but can be separated from the vesicles by gel filtration.

The most probable interaction of heparin with phosphatidylcholine is between the quaternary ammonium group of the phospholipid and the sulphate group of the glycosaminoglycan. Thus, in this regard, Hauser & Phillips (1976), studying the conformation of the phosphatidylcholine polar groups in charged vesicles with paramagnetic probes, showed that the $N(CH_3)_3^+$ group of phosphatidylcholine binds most readily to anions high in the chaotropic series, such as RSO_4^-. In addition, they showed that the anionic phosphate group is far enough from the cationic polar head of the phospholipid, and deeply embedded in the bilayer, to avoid repulsion of anionic external ligands.

A 13000–14000-M_r heparin molecule has an average number of sulphate groups (theoretically available for binding) of about 60. When all are saturated by phosphatidylcholine molecules, a large increase in M_r (about 42000) should occur. This does not seem the case, from measurements of the size of the complex by gel filtration, and therefore we assume that only a few sterically favoured groups of heparin bind phosphatidylcholine, forming a partially saturated complex with an amphiphilic nature and a differential solubility in aqueous or organic solvents depending on the ratio between sulphate groups that have interacted or not interacted with phosphatidylcholine. This could be responsible for the variable yield of plasma heparin in the inorganic phases.

The biological significance of the complexing of heparin with a major membrane phospholipid remains unknown. At the plasma level it could represent a mechanism of abstraction and storage of a highly active compound. However, at the cellular level it could be involved in some important activities of heparin in the vascular system other than anticoagulant, e.g. the angiogenetic and the platelet proaggregating effects.

Heparin is well known to stimulate vessel growth (Taylor & Folkman, 1982) and to act as a chemoattractant of capillary endothelial cells *in vitro* (Azizkhan *et al.*, 1980; Alessandri *et al.*, 1983); in addition, the proaggregating effect on platelets is confirmed by a variety of evidence (Ruggiero *et al.*, 1984).

The interaction of heparin with the cationic

polar head of phosphatidylcholine could induce abstraction of phospholipids by stripping them off as well as changes in the membrane bilayer assembly. It seems that platelets and endothelial cells are very sensitive to this kind of membrane stimulus: heparin triggers the phosphatidylinositol cycle in platelets (Ruggiero *et al.*, 1984) as well as in capillary-endothelial-cell cultures (V. Chiarugi & M. Ruggiero, unpublished work). The mechanisms of such stimulatory activity is unknown, but the stripping off and the consequent local lowering of the concentration of phosphatidylcholine could be involved; thus Irvine *et al.* (1984) revealed that when the phosphatidylcholine/phosphatidylethanolamine concentration ratio in artificial membranes is lowered under a critical value incompatible with the bilayer assembly, polyphosphoinositides are much more rapidly hydrolysed by the specific phosphodiesterase.

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